JOURNAL OF BACTERIOLOGY, June 1995, p. 3596–3600
0021-9193/95/$04.00+0

Vol. 177, No. 12

Physiological and Biochemical Analysis of the Effects of Alkaline Phosphatase Overproduction in *Escherichia coli*

HIROSHI KADOKURA, KEI WATANABE, KAZUHIDE TSUNEIZUMI, KOJI YODA, AND MAKARI YAMASAKI*

Department of Biotechnology, University of Tokyo, Yayoi, Bunkyo-ku, Tokyo 113, Japan

Received 25 July 1994/Accepted 7 April 1995

Overexpression of the *Escherichia coli phoA* gene, coding for alkaline phosphatase (PhoA), on multicopy plasmids caused a severe defect in the precursor processing (secretion) of PhoA, β-lactamase, and the outer membrane protein OmpA. This secretion defect continued even after the repression of *phoA* expression, indicating that protein secretion was irreversibly impaired in cells. Among the secretory proteins, only OmpA gradually secreted posttranslationally. The inverted inner membrane vesicles prepared from cells with the secretion defect showed appreciably reduced translocation activity in vitro. But the membrane vesicles retained the ability to generate a proton motive force which, together with ATP, is essential as an energy source for the efficient secretion of proteins in *E. coli*. An appreciable amount of incompletely translocated PhoA molecules was detected in the inner membranes of cells with the secretion defect.

Alkaline phosphatase (PhoA), encoded by the *phoA* gene of *Escherichia coli*, is a well-known periplasmic enzyme. The enzyme is synthesized as a precursor monomer with a signal peptide at the amino-terminal end. After translocation into the periplasmic space, the signal peptide is removed and two of the resulting mature monomers dimerize, in the presence of four Zn^{2+} ions and two Mg^{2+} ions, to produce an active enzyme (10). The synthesis of PhoA is regulated by a two-component regulatory system composed of the products of the *phoB* and *phoR* genes (36). The expression of the *phoA* gene is induced under phosphate-limited conditions, allowing regulated expression (33). The *phoA* gene has an efficient expression system, as the PhoA protein represents 6% of the newly synthesized protein after induction (33).

We cloned the *phoA* gene to make a secretion vector system with the promoter and the signal sequence of *phoA* (39, 40). During the study of the expression of the *phoA* gene on multicopy plasmids, we observed that about 80% of the *phoA* gene product (the sum of the mature and the precursor forms of PhoA) failed to be secreted and formed insoluble inclusion bodies in the cytoplasm (9, 40). The accumulation of the precursor molecules in this manner in secretory-protein-overproducing *E. coli* has been reported for a periplasmic phosphate-binding protein (23) and an outer membrane protein, OmpA (5). Precursor accumulation of this type has commonly been explained in terms of export site saturation: when a secretory protein is produced above the secretion capacity of a cell, the export site becomes saturated and the excess protein accumulates in the cytoplasms of the cells as a precursor species (30).

We have recently shown that PhoA secretion can be enhanced greatly by mutations decreasing (and thus optimizing) the synthetic rates of the *phoA* gene product (9). This is contrary to what the export site saturation model predicts: the level of secretion should be at a plateau when the level of expression increases beyond a certain point. This result suggests that the overproduction of PhoA, even though it is a normal secretory protein of *E. coli*, causes a secretion defect in *E. coli* cells. These findings prompted us to investigate the effects of PhoA overproduction in *E. coli*. Our data show that the overproduction of the *phoA* gene product irreversibly impairs the ability of the cells to secrete and suggest that the defect is an event at the site of the inner membrane.

Effect of PhoA overproduction on the secretion kinetics of PhoA, OmpA, and β-lactamase proteins. It has been previously demonstrated that the overproduction of the *phoA* gene product by growing *E. coli* strains carrying a multicopy plasmid containing the *phoA* gene in phosphate-limiting medium caused the accumulation of the precursor form of the PhoA protein in addition to its mature form (9). This defect was evident after induction of *phoA* expression for more than 1 h. About 80% of the newly synthesized PhoA protein localized in the cytoplasm in a precursor form (9, 40). Similar results were obtained when overproduction of the *phoA* gene product was induced by growing *E. coli* strains carrying plasmid pHK152 (*phoA* under *tacI* control [3, 9]) in the presence of 0.1 mM IPTG (isopropyl-β-D-thiogalactopyranoside), indicating that the secretion defect does not depend on the expression system employed (9). Plasmid pHK152 is a derivative of pUC18 carrying *phoA* under the control of the *tacI* promoter (3, 9).

To know the effect of PhoA overproduction on protein secretion, the secretion kinetics of proteins were studied by pulse-chase experiments and immunoprecipitations. *E. coli* JM103 (38) carrying pHK152 was grown at 37°C in M9 minimal medium (22) supplemented with 10 μg of thiamine per ml and 50 μg of each amino acid except L-methionine per ml. When the optical density of the culture at 550 nm reached 0.2, the expression of the multicopy *phoA* gene from the *tacI* promoter was induced by the addition of 0.1 mM IPTG. The induction was continued for 1 h. Before the secretion kinetics were measured, the expression of *phoA* on the multicopy plasmid pHK152 was reduced to the level of uninduced cells by growing cells in fresh M9 minimal medium without IPTG for another 30 min because we wanted to examine the secretion defect at the normal level of production of PhoA. The postincubation period was indeed sufficient to reduce the level of expression of the *phoA* gene of the induced cells to that of the uninduced cells as evidenced in Fig. 1A (compare the levels of incorporation of L-[^{35}S]methionine into PhoA proteins in IPTG-induced and uninduced cells). After a postincubation period of 30 min, the cells were labeled with 20 μCi of L-[^{35}S]methionine (1,000 Ci/mmol; Amersham) per ml for 30 s.

* Corresponding author. Mailing address: Department of Biotechnology, University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113, Japan. Phone: 81-3-3812-2111, ext. 5161. Fax: 81-3-5689-7294.

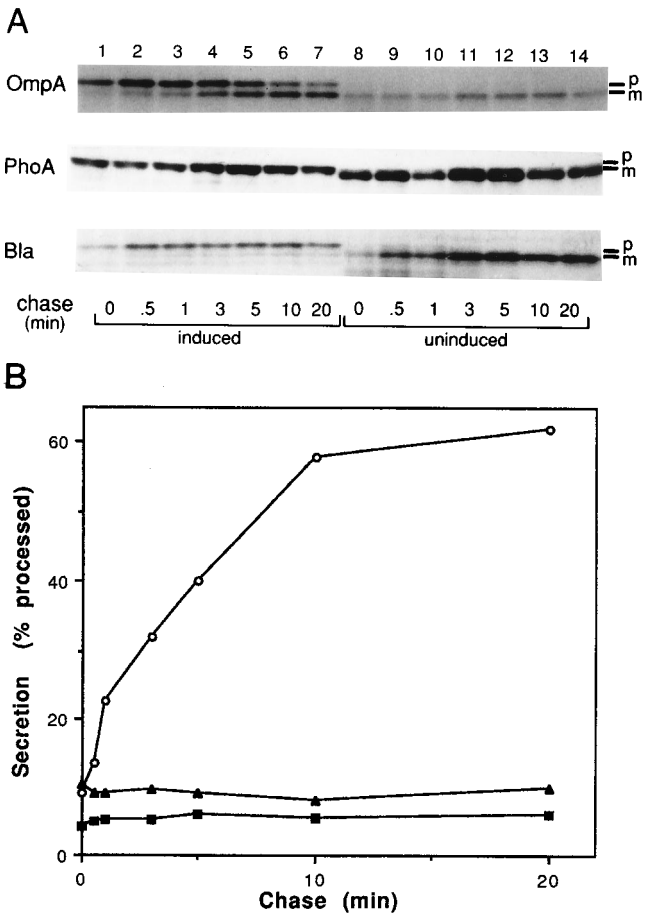

FIG. 1. Secretion kinetics of proteins in cells after induction of multicopy *phoA*. (A) Cells of strain JM103 carrying pHK152 were grown in M9 minimal medium (10 ml) (22) supplemented with 10 μg of thiamine per ml and 50 μg of each amino acid except L-methionine per ml. When the optical density at 550 nm reached 0.2, IPTG was added to a final concentration of 0.1 mM (in lanes 1 to 7) or was not added (lanes 8 to 14). After 1 h, the cells were collected and transferred to fresh M9 minimal medium without IPTG and growth was continued for another 30 min. The cells were then pulse-labeled with L-[^{35}S]methionine for 30 s and chased with excess unlabeled methionine. At the indicated time points, the samples were removed, the chase was terminated, the protein products were divided into three portions, and each aliquot was immunoprecipitated with an appropriate antiserum and analyzed by SDS-polyacrylamide gel electrophoresis and fluorography. The positions of the precursor (p) and mature form (m) of each protein species are indicated at the right. (B) The secretion kinetics (percentages processed) of OmpA (○), PhoA (■), and Bla (▲) after the induction of multicopy *phoA* were measured by quantitating the intensities of bands corresponding to the precursor and the mature forms of these proteins in the fluorograms in lanes 1 to 7 in panel A with an automatic computing densitometer (model AD-18; Gelman Sciences, Inc.).

Chase with unlabeled L-methionine was done by the addition of 100 μg of L-methionine per ml. The pulse-chase samples were immediately precipitated with trichloroacetic acid. The resultant precipitates were solubilized and divided into three portions, and each aliquot was immunoprecipitated with an appropriate antiserum as described previously (7). The proteins tested were the outer membrane protein OmpA and the periplasmic proteins PhoA and β-lactamase (Bla). PhoA and Bla are encoded by plasmid pHK152. Antiserum against Bla has been described (8). Antiserum against OmpA was generously provided by S. Mizushima, Institute of Molecular and Cellular Biosciences, University of Tokyo. Antiserum against PhoA has been described (9). IgGsorb (Enzyme Center Inc., Malden, Mass.) was used to collect the immune complex that formed. Immunoprecipitated polypeptides were analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (13) and fluorography. The fraction of each polypeptide in its mature form was calculated following the quantification of mature and precursor proteins with an automatic computing densitometer (model AD-18; Gelman Sciences, Inc., Ann Arbor, Mich.). At the time of pulse-labeling, the optical densities of the induced culture and the uninduced culture at 550 nm were 0.60 and 0.68, respectively. The sampling volumes for the induced culture and for the uninduced culture were 0.34 and 0.30 ml, respectively. Thus, we can compare the levels of incorporation of label into a protein in samples containing the same amount of cells. The growth rate of the induced cells, as evidenced by the incorporation of label into an acid-insoluble fraction, was about one-half of that of the uninduced cells at the time of pulse-labeling (data not shown), indicating that induction of the multicopy *phoA* gene for 1 h caused a growth defect. As shown in Fig. 1A, all three proteins tested were rapidly and efficiently secreted in cells grown in the absence of IPTG, as indicated by the finding that the great majority of proteins precipitated at the 0-min chase points were found in their mature forms. However, in the IPTG-induced cells, all proteins tested exhibited a defect in signal sequence processing. About 90% of the newly synthesized proteins were found in their precursor forms at the 0-min chase points, exhibiting an obvious secretion defect (Fig. 1B). This defect was most pronounced with the PhoA and Bla proteins. Even at the longer chase times, the majority of the PhoA and Bla proteins remained in their precursor forms, indicating that the secretion of these proteins was impaired irreversibly in the IPTG-induced cells. In marked contrast, OmpA protein was secreted and processed posttranslationally (62% of the protein was processed at 20 min).

It should be noted that OmpA was expressed at an appreciably higher level in the induced culture than in the uninduced culture, while Bla was expressed at a rate roughly proportional to the growth rate (Fig. 1A). Further experiments are needed to define the nature of the increased expression of OmpA in the induced culture.

Translocation assay with membrane vesicles isolated from cells with the secretion defect. To localize the site of the secretion defect caused by the overproduction of PhoA, we isolated inverted *E. coli* membrane vesicles from strain JM103 (38) carrying pKI-5 grown under the phosphate-limited condition for 1 h, during which time a severe defect in protein secretion like that shown in Fig. 1 was observed (data not shown). Plasmid pKI-5 is a derivative of pBR322 carrying the *phoA* gene (9). Induction by phosphate limitation was employed in this case because, for an unknown reason, sufficient expression of PhoA was not obtained by induction from the *tacI* promoter, with 0.1 or 1 mM IPTG, when the culture volume was increased to 1 liter to isolate inverted membranes. As a control, we used inverted membrane vesicles prepared from strain JM103 carrying pBR322 grown under the phosphate-limited condition for 1 h. In this case, the expression of a single chromosomal copy of the *phoA* gene was induced. To induce the expression of *phoA* from its own promoter, medium 121 (9) supplemented with 100 μg of L-leucine per ml and with either 0.64 mM sodium phosphate (referred to as high-phosphate medium) or 0.064 mM sodium phosphate (referred to as low-phosphate medium) was used. For the expression of PhoA, *E. coli* cells grown in the high-phosphate medium until the optical density at 550 nm reached 0.4 were transferred to the low-phosphate medium and incubated for 1 h at 37°C. Then, purified inverted membrane vesicles were prepared from *E.*

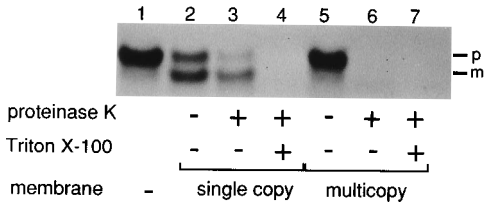

FIG. 2. Translocation assay with membrane vesicles isolated from cells upon overproduction of PhoA. Expression of the *phoA* gene in *E. coli* JM103(pBR322) and JM103(pKI-5) was induced in low-phosphate medium. After 1 h, the cells were harvested, and purified inverted membrane vesicles were prepared. Vesicles from JM103(pBR322) (lanes 2 to 4) and from JM103(pKI-5) (lanes 5 to 7) were then subjected to a protein translocation assay with cleavable OmpF-Lpp (37). Lane 1 contained no membrane vesicles. After translocation, the samples for lanes 3, 4, 6, and 7 were treated with proteinase K in the presence (lanes 4 and 7) or absence (lanes 3 and 6) of 1% Triton X-100. All samples were then analyzed by polyacrylamide gel electrophoresis and subsequent fluorography. The positions of the precursor (p) and mature form (m) of cleavable OmpF-Lpp are indicated at the right.

coli according to the method of Yamada et al. (35). Briefly, spheroplasts formed in the presence of lysozyme, EDTA, and sucrose were passed through a French pressure cell, and the inverted membrane vesicles formed were fractionated by isopycnic sucrose gradient centrifugation. The relative activities of NADH oxidase in the fractions were determined as described previously (11), and the fraction showing the highest relative activity was used as purified inverted membrane vesicles. The amount of membrane vesicles was expressed as that of protein, which was determined by the method of Lowry et al. (16). These membrane vesicles were then tested for the ability to translocate proteins by using an in vitro translocation system with a model substrate, cleavable OmpF-Lpp (37).

In vitro transcription of the gene for cleavable OmpF-Lpp was performed with SP6 RNA polymerase by using *Hin*dIII-digested plasmid pK105 as a template as previously described (37). The translation reaction was carried out with S100 fraction (the 100,000 × *g* supernatant of *E. coli* cell lysate) prepared from *E. coli* K003 (Lpp$^-$ Δ*uncB-C*-Tn*10*) in the presence of L-[^{35}S]methionine (specific activity, 1,000 Ci/mmol) as previously described (35). Plasmid pK105 carried an *ompF-lpp* chimeric gene that codes for a cleavable signal sequence-containing OmpF-Lpp chimeric protein under the control of the SP6 promoter (37). *E. coli* K003 and plasmid pK105 were generous gifts of S. Mizushima. The translocation reaction was carried out as described previously (35) with the inverted membrane vesicles purified as described above. The reaction mixture was then treated with proteinase K (Boehringer Mannheim), and the translocated protein, which was proteinase K resistant, was detected on SDS-polyacrylamide gels by fluorography. The amounts of protein in the individual bands on gels were estimated densitometrically with an automatic computing densitometer (model AD-18; Gelman Sciences, Inc.). The efficiency of translocation was estimated from the intensities, on fluorograms, of protein bands before and after the proteinase K treatment. For the estimation, the numbers of methionine residues in the precursor and the mature forms of the secretory proteins were taken into consideration as described previously (35). Figure 2 shows that the translocation activity of inverted membrane vesicles isolated from cells after overproduction of PhoA was reduced appreciably (lane 6). Quantification of the autoradiograms showed that the vesicles possessed 10% of the translocation activity of membrane vesicles prepared from cells carrying only a single chromosomal copy of the *phoA* gene, grown under identical conditions (Fig. 2, lane 3). This defect was also observed when uncleavable OmpF-Lpp (37) was used as a substrate (data not shown). These results suggest that the secretion defect is an event at the site of the inner membrane.

It has been well established that both ATP and a proton motive force ($\Delta\tilde{\mu}H^+$) are required for protein secretion in *E. coli* cells, even though the extent of the requirement for $\Delta\tilde{\mu}H^+$ varies with the proteins tested (35). As the inner membrane vesicles prepared from the PhoA-overproducing cells showed reduced secretion ability, there is a possibility that the ability of the membrane to generate $\Delta\tilde{\mu}H^+$ has been lost in the cells with the secretion defect. To test this, the ability of these membrane vesicles to generate $\Delta\Psi$ and ΔpH, which together constitute $\Delta\tilde{\mu}H^+$, was examined with oxonol V and quinacrine as fluorescence probes, respectively, essentially as described previously (35). The results showed that the levels of both $\Delta\Psi$ and ΔpH produced by the membrane vesicles prepared from the cells induced by multicopy *phoA* were comparable to the levels produced by the membrane vesicles prepared from the cells induced by single-copy *phoA* (data not shown). This suggests that some function of the inner membrane, other than the generation of $\Delta\tilde{\mu}H^+$, was impaired in the inner membranes of the cells with the secretion defect.

Accumulation of the precursor and mature forms of PhoA in the inner membranes of cells with the secretion defect. Several studies involving fusions between a secretory protein and a cytoplasmic protein showed that the secretion defect is accompanied by the accumulation of the fusion protein in the inner membrane (27, 32). To determine whether such molecules could be detected in the inner membrane vesicles, the proteins of these membranes were analyzed by Western blotting (immunoblotting) with an anti-PhoA antiserum. It should be noted that after the overproduction of PhoA, the majority of molecules of the precursor form accumulated in the cytoplasm while the majority of molecules of the mature form accumulated in the periplasm (9, 40).

E. coli PhoA is synthesized as a precursor monomer with a signal peptide at the N terminus. After translocation into the periplasmic space, the signal peptide is removed, the disulfide bonds are formed with the aid of DsbA (1), and two of the mature monomers dimerize, in the presence of four Zn^{2+} ions and two Mg^{2+} ions, to yield an active enzyme (2, 10). The dimerization of the monomers is absolutely required for the enzyme activity, and it occurs only when the monomers are secreted into the periplasm (2, 18). Additionally, the dimer, once formed, is highly resistant to detergents, reductants, and proteolytic enzymes. Experimentally, it retains almost full activity even in the presence of 5% (wt/vol) SDS or 4% (vol/vol) β-mercaptoethanol (19). Usually, heat treatment of the enzyme—incubation at 95°C for more than 10 min— is necessary to dissociate the dimer into monomers. For refined analysis of the PhoA molecules in the inner membranes, we made use of these special features of PhoA.

Figure 3 shows the results of Western blotting, with a rabbit anti-PhoA antiserum, of the inner membrane proteins (20 μg) prepared from cells 1 h after expression of multicopy or single-copy *phoA* as described in the legend to Fig. 2. Before SDS-polyacrylamide gel electrophoresis, the membrane samples either were heated at 95°C for 10 min in the Laemmli sample buffer (13) or were not heated. After SDS-polyacrylamide gel electrophoresis, the gel was incubated at 90°C for 3 min in 50 mM Tris-HCl (pH 8.3) buffer containing 1% SDS and 4% β-mercaptoethanol and was washed twice by incubation at 30°C for 30 min in a transfer buffer (20 mM Tris-HCl [pH 8.3], 154 mM glycine, 0.1% SDS, 20% methanol). These steps were necessary to transfer the proteins, corresponding to the PhoA dimer, to a nitrocellulose filter. The gel was then subjected to

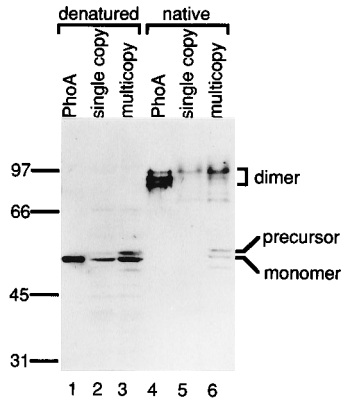

FIG. 3. Accumulation of precursor and mature PhoA molecules in inner membrane vesicles. Membrane vesicles were prepared from cells 1 h after the induction of a multicopy or single-copy *phoA* gene as described in the legend to Fig. 2. PhoA protein (20 μg) in the inner membrane vesicles was examined by Western blotting with a rabbit anti-PhoA antiserum. Lanes 2 and 5 contain membrane vesicles from JM103(pBR322); lanes 3 and 6 contain membrane vesicles from JM103(pKI-5). Lanes 1 and 4 contain purified PhoA (0.2 μg) as a control. Before electrophoresis, samples were not incubated (native) or were incubated at 95°C for 10 min (denatured) in Laemmli sample buffer (13). The positions of marker proteins are indicated on the left (in kilodaltons). The positions of the PhoA dimer, precursor, and mature monomer are indicated on the right. Bands migrating at 66 and 45 kDa in the denatured samples (lanes 2 and 3) are from nonspecific proteins. Bands at 68 kDa in the native samples (lanes 5 and 6) and at 46 kDa in the multicopy samples (lanes 3 and 6) are derived from the proteolytic degradation of PhoA. The specificity of the antiserum was checked with the cell lysate of *E. coli* YK537 (*phoA8* [9]) grown in low-phosphate medium.

Western blotting with a rabbit anti-PhoA antiserum, as described previously (9). The specificity of the antiserum used was checked with a cell lysate of *E. coli* YK537 (*phoA8* [9]) grown in low-phosphate medium. The dimer form of PhoA is found in the membrane vesicles prepared from the cells 1 h after the expression of single-copy or multicopy *phoA* (Fig. 3, lanes 4 to 6). These PhoA dimers in the periplasm must have been trapped in the inverted membrane vesicles when the inverted membrane vesicles were formed from the inner membrane on a French pressure cell. Triplet bands of PhoA dimers represent isozymes generated by the trimming of the N-terminal arginine residues presumably catalyzed by the *iap* gene product (6). When the samples were heated before electrophoresis, these PhoA dimers disappeared, leaving mature monomers (Fig. 3, lanes 1 to 3). These results are consistent with the previous results showing that the PhoA dimer is resistant to detergents and reductant and that heat treatment is necessary to dissociate the dimer into the monomers (19). In marked contrast, the mature monomer and the precursor were detected only in membrane vesicles prepared from the cells with the secretion defect (Fig. 3, lane 6). Even when five times as much sample as that in lane 5 of Fig. 3 was loaded in a lane, we could not detect the monomer forms of PhoA in membrane vesicles from the cells that produce PhoA at a normal level (data not shown). Considering the detection limit on the Western blot as evidenced by experiments with purified PhoA as a standard (data not shown), the monomer forms of PhoA contained in the membrane vesicles from cells of the strain that produces PhoA at a normal level are less than 1/40 of those in the vesicles from the strain that overproduces PhoA. The number of these molecules (the precursor and the mature monomers) in the inner membranes of the cells with the secretion defect was estimated, by Western blotting with purified PhoA protein as a standard (data not shown), to be about 600 molecules per single cell (20).

We then examined if these PhoA molecules, accumulated in the inner membrane of the cells with the secretion defect, could translocate within the membrane posttranslationally by incubating the inner membrane vesicles in the presence of ATP and NADH. Even when the membrane vesicles were incubated in the presence of ATP and NADH, we could not detect any change in the amount of the mature monomer on the precursor (data not shown). This suggests that PhoA molecules, once they accumulated in the inner membrane, could not be translocated posttranslationally.

The results reported above show that the overproduction of PhoA, even though it is a normal secretory protein of *E. coli*, impairs the ability of cells to secrete and suggest that the secretion defect is an event at the site of the inner membrane. A similar defect in protein secretion has been reported for cells synthesizing fusions between a secretory protein and a cytoplasmic protein, e.g., β-galactosidase (LacZ) fusions (26, 31). In such cells the LacZ fusion was observed to accumulate in the cytoplasmic membrane (27, 32), and the secretion defect has been explained in terms of the secretion incompatibility of the LacZ moiety (14). The secretion initiated by the signal in the secretory protein moiety of the fusion protein is interrupted by the LacZ moiety, resulting in the jamming of the secretion pathway. Then why does the overproduction of PhoA, a normal secretory protein of *E. coli*, cause a secretion defect? At present we have no absolute answer to this question. There is a possibility that the continuous inflow of protein molecules to be secreted is inhibitory at the translocation site (21) and somehow causes jamming. Indeed, we detected precursor and mature monomer forms of PhoA in the inner membrane vesicles of the cells with the secretion defect. The number of these molecules per single cell was estimated to be about 600. This agrees roughly with the formerly estimated number for the translocation machinery existing in one *E. coli* cell (20). Thus, these PhoA molecules may be trapped at the translocation site. Further experiments are necessary to confirm this point.

Our present results, together with our results reported previously (9), show that synthesis of PhoA above a certain level is required to cause the secretion defect. Relationships between the level of protein synthesis and secretion, such as this, have been described. Shiba et al. (29) and Lee and Beckwith (15) have characterized several suppressors of a *secA*(Ts) or a *secY*(Ts) mutant and have shown that they are the mutations in genes involved in or affecting protein synthesis. Lee and Beckwith (15) showed that a very low concentration of chloramphenicol, which does not stop but does slow down protein synthesis, can suppress the temperature sensitivity and secretion defect of a *secY*(Ts) mutant and the cold sensitivity and secretion defect of a *secD*(Cs) mutant. Freudl et al. (4) showed that two nonsecretory proteins, dihydrofolate reductase and β-galactosidase could be translocated across the plasma membrane of *E. coli* with the aid of the signal sequence of pro-OmpA only when the amounts synthesized were sufficiently small. These results suggest that the secretion defect can occur at different levels of protein production depending on both the secretion capacity of the cell and the protein produced, whether it is a normal secretory protein or a fusion between a secretory and a cytoplasmic protein.

The present in vivo studies show that the extent of the secretion defect differed with the protein tested; upon overproduction of PhoA, the secretion of PhoA and Bla was abolished almost completely whereas OmpA secreted posttranslationally. The difference in the extent of the secretion defect

might be correlated with the different preferences (of each preprotein) for chaperonic factors. From some studies (17, 24, 34) it appears that the secretion of PhoA and Bla involves Ffh (P48) and 4.5S RNA, which together constitute a signal recognition particle-like complex in *E. coli* (25, 28). In contrast, the secretion of OmpA involves SecB (12, 28). Since in vivo results (Fig. 1) show that a small portion (about 10%) of the newly synthesized PhoA and Bla proteins was secreted even after the overproduction of PhoA, it is possible that most, but not all, of the translocation sites were impaired (some translocation sites still remained intact) after the overproduction of PhoA and that pro-OmpA, which might somehow remain competent for secretion possibly by the SecB function, was translocated posttranslationally through some translocation sites which remained intact.

We are grateful to S. Mizushima for the kind gift of the *E. coli* strain, the plasmid, and the antiserum used and to H. Tokuda for helpful advice.

This work was performed as a part of the Research and Development Projects of Industrial Science and Technology Frontier Program supported by NEDO (New Energy and Industrial Technology Development Organization).

REFERENCES

1. **Bardwell, J. C. A., K. McGovern, and J. Beckwith.** 1991. Identification of a protein required for disulfide bond formation *in vivo*. Cell **67:**581–589.
2. **Boyd, D., C.-D. Guan, S. Willard, W. Wright, K. Strauch, and J. Beckwith.** 1987. Enzymatic activity of alkaline phosphatase precursor depends on its cellular location, p. 89–93. *In* A. Torriani-Gorini, F. G. Rothman, S. Silver, A. Wright, and E. Yagil (ed.), Phosphate metabolism and cellular regulation in microorganisms. American Society for Microbiology, Washington, D.C.
3. **De Boer, H. A., L. J. Comstock, and M. Vassar.** 1983. The *tac* promoter: a functional hybrid derived from the *trp* and *lac* promoters. Proc. Natl. Acad. Sci. USA **80:**21–25.
4. **Freudl, R., H. Schwarz, S. Kramps, I. Hindennach, and U. Henning.** 1988. Dihydrofolate reductase (mouse) and β-galactosidase (*Escherichia coli*) can be translocated across the plasma membrane of *E. coli*. J. Biol. Chem. **263:**17084–17091.
5. **Freudl, R., H. Schwarz, Y. D. Stierhof, K. Gamon, I. Hindennach, and U. Henning.** 1986. An outer membrane protein (OmpA) of *Escherichia coli* K-12 undergoes a conformational change during export. J. Biol. Chem. **261:**11355–11361.
6. **Ishino, Y., H. Shinagawa, K. Makino, M. Amemura, and A. Nakata.** 1987. Nucleotide sequence of the *iap* gene, responsible for alkaline phosphatase isozyme conversion in *Escherichia coli*, and identification of the gene product. J. Bacteriol. **169:**5429–5433.
7. **Ito, K., P. J. Bassford, Jr., and J. Beckwith.** 1981. Protein localization in *E. coli*: is there a common step in the secretion of periplasmic and the outer-membrane proteins? Cell **24:**707–717.
8. **Kadokura, H., K. Yoda, M. Imai, and M. Yamasaki.** 1990. Production and secretion in *Escherichia coli* of hepatitis B virus pre-S2 antigen as fusion proteins with β-lactamase. Appl. Environ. Microbiol. **56:**2742–2747.
9. **Kadokura, H., K. Yoda, S. Watanabe, Y. Kikuchi, and M. Yamasaki.** 1994. Enhancement of protein secretion by optimizing protein synthesis: isolation and characterization of *Escherichia coli* mutants with increased secretion ability of alkaline phosphatase. Appl. Microbiol. Biotechnol. **41:**163–169.
10. **Kim, E. E., and H. W. Wyckoff.** 1991. Reaction mechanism of alkaline phosphatase based on crystal structures: two-metal ion catalysis. J. Mol. Biol. **218:**449–464.
11. **Kim, Y. J., S. Mizushima, and S. Tokuda.** 1991. Fluorescence quenching studies on the characterization of energy generated at the NADH: quinone oxidoreductase and quinol oxidase segments of marine bacteria. J. Biochem. **109:**616–621.
12. **Kumamoto, C. A., and J. Beckwith.** 1985. Evidence for specificity at an early step in protein export in *Escherichia coli*. J. Bacteriol. **163:**267–274.
13. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
14. **Lee, C., P. Li, H. Inouye, E. R. Brickman, and J. Beckwith.** 1989. Genetic studies on the inability of β-galactosidase to be translocated across the *Escherichia coli* cytoplasmic membrane. J. Bacteriol. **171:**4609–4616.
15. **Lee, C. A., and J. Beckwith.** 1986. Suppression of growth and protein secretion defects in *Escherichia coli secA* mutants by decreasing protein synthesis. J. Bacteriol. **166:**878–883.
16. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193:**265–275.
17. **Lurink, J., and B. Dobberstein.** 1994. Mammalian and *Escherichia coli* signal recognition particles. Mol. Microbiol. **11:**9–13.
18. **Manoil, C., J. J. Mekalanos, and J. Beckwith.** 1990. Alkaline phosphatase fusions: sensors of subcellular location. J. Bacteriol. **172:**515–518.
19. **Mather, I. H., and T. W. Keenan.** 1974. The stability of alkaline phosphatase in sodium dodecyl sulfate. FEBS Lett. **44:**79–82.
20. **Matsuyama, S., Y. Fujita, K. Sagara, and S. Mizushima.** 1992. Overproduction, purification and characterization of SecD and SecF, integral membrane components of the protein translocation machinery of *Escherichia coli*. Biochim. Biophys. Acta **1122:**77–84.
21. **Matsuyama, S.-I., Y. Fujita, and S. Mizushima.** 1993. SecD is involved in the release of translocated secretory proteins from the cytoplasmic membrane of *Escherichia coli*. EMBO J. **12:**265–270.
22. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
23. **Pages, J.-M., J. Anba, A. Bernadac, H. Shinagawa, A. Nakata, and C. Lazdunski.** 1984. Normal precursors of periplasmic proteins accumulated in the cytoplasm are not exported post-translationally in *Escherichia coli*. Eur. J. Biochem. **143:**499–505.
24. **Philips, G. J., and T. J. Silhavy.** 1992. The *E. coli ffh* gene is necessary for viability and efficient protein export. Nature (London) **359:**744–746.
25. **Poritz, M. A., H. D. Bernstein, K. Strub, D. Zopf, H. Wilhelm, and P. Walter.** 1990. An *E. coli* ribonucleoprotein containing 4.5S RNA resembles mammalian signal recognition particle. Science **250:**1111–1117.
26. **Pugsley, A. P.** 1993. The complete general secretory pathway in gram-negative bacteria. Microbiol. Rev. **57:**50–108.
27. **Rasmussen, B. A., V. A. Bankaitis, and P. J. Bassford, Jr.** 1984. Export and processing of MalE-LacZ hybrid proteins in *Escherichia coli*. J. Bacteriol. **160:**612–617.
28. **Ribes, V., K. Romisch, A. Giner, B. Dobberstein, and D. Tollervey.** 1990. The *E. coli* 4.5S RNA is part of a ribonucleoprotein particle that has properties related to signal recognition particle. Cell **63:**591–600.
29. **Shiba, K., K. Ito, and T. Yura.** 1984. Mutation that suppresses the protein export defect of the *secY* mutation and causes cold-sensitive growth of *Escherichia coli*. J. Bacteriol. **160:**696–701.
30. **Simonen, M., and I. Palva.** 1993. Protein secretion in *Bacillus* species. Microbiol. Rev. **57:**109–137.
31. **Stader, J. A., and T. J. Silhavy.** 1990. Engineering *Escherichia coli* to secrete heterologous gene products. Methods Enzymol. **185:**166–187.
32. **Tommassen, J., and T. de Kroon.** 1987. Subcellular localization of a PhoA-LacZ fusion protein in *E. coli* by protease accessibility experiments reveals an inner-membrane-spanning form of the protein. FEBS Lett. **221:**226–230.
33. **Wanner, B. L.** 1987. Phosphate regulation of gene expression in *Escherichia coli*, p. 1326–1333. *In* F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology. American Society for Microbiology, Washington, D.C.
34. **Wolin, S. L.** 1994. From the elephant to *E. coli*: SRP-dependent protein targeting. Cell **77:**787–790.
35. **Yamada, H., H. Tokuda, and S. Mizushima.** 1989. Proton motive force-dependent and -independent protein translocation revealed by an efficient *in vitro* assay system of *Escherichia coli*. J. Biol. Chem. **264:**1723–1728.
36. **Yamada, M., K. Makino, M. Amemura, H. Shinagawa, and A. Nakata.** 1989. Regulation of the phosphate regulon of *Escherichia coli*: analysis of mutant *phoB* and *phoR* genes causing different phenotypes. J. Bacteriol. **171:**5601–5606.
37. **Yamane, K., S. Matsuyama, and S. Mizushima.** 1988. Efficient *in vitro* translocation into *Escherichia coli* membrane vesicles of a protein carrying an uncleavable signal peptide. J. Biol. Chem. **263:**5368–5372.
38. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33:**103–119.
39. **Yoda, K., Y. Kikuchi, M. Yamasaki, and G. Tamura.** 1980. Cloning of the structural gene of alkaline phosphatase of *Escherichia coli* K12. Agric. Biol. Chem. **44:**1213–1214.
40. **Yoda, K., K.-I. Tachibana, S. Watanabe, K. Yamane, M. Yamasaki, and G. Tamura.** 1987. Secretion to periplasm of foreign proteins in *Escherichia coli* by aid of the *phoA*-derived secretion vector Psi, p. 99–104. *In* A. Torriani-Gorini, F. G. Rothman, S. Silver, A. Wright, and E. Yagil (ed.), Phosphate metabolism and cellular regulation in microorganisms. American Society for Microbiology, Washington, D.C.